REMARKS

1. Information Disclosure Statement

Applicants will shortly provide a further copy of references cited in the information disclosure statement filed December 7, 2001 under separate cover. Because the references were provided with the original submission as evidenced by a stamped postcard (previously submitted), and subsequently, and have been apparently lost in the PTO on both occasions, it is requested that the references be considered with effect from their original submission date of December 7, 2001. It is further noted that, in the Response dated July 22, 2002, applicants requested the Examiner to call if she was still unable to access the references, but in fact applicants did not learn of this until the present office action.

2. Restriction Requirement

The Examiner maintains the restriction requirement between groups I and II on the basis that Choo et al. demonstrates the special technical feature of a polynucleotide. Applicants maintain traverse. The special technical feature unifying the Group I and II claims is the selection of a quadruplet of bases within a target sequence, for use in the design of a binding protein. For the reasons discussed in the previous response, and below, such is not disclosed by Choo et al. Therefore, applicants request that the group II claims be rejoined to the group I claims.

With respect to division between groups II and III, the Examiner maintains that the use of nucleic acids in a hybridization assay is not a throw-away utility but rather is often necessary for the development of new compounds and procedures. However, it is not clear to Applicants how the claimed polynucleotides, which encode non-naturally-occurring, engineered proteins, could be used in any type of hybridization assay. For example, it is not clear to what the nucleic acids would hybridize. Accordingly, the

alleged utility asserted by the office action fails to fulfill the PTO's specificity prong of the "specific, substantial and credible" requirement under 35 USC 101.

3. Obviousness-Type Double Patenting

Claims 1-24 and 32 stand rejected for obviousness type double patenting over claims 1-12 and 30 of copending USSN 09/424,488. The Examiner notes that when base 4 is A, the current application specifies Glu, Asn or Val, whereas the copending application specifies Gln, which differs from Glu only by the penultimate R group. The Examiner also notes that when base 4 is C, the current application specifies Ser, Thr, Val, Ala, Glu or Asn, whereas the copending application indicates any amino acid.

Applicants disagree that the differences the Examiner has identified are so minor as to support obviousness-type double patenting. The test of obviousness-type double patenting is whether any claim of the cited application renders any of the present claims obvious. See MPEP 804 B.1. Although the Office Action states that Glu and Gln differ only by the terminal R group, this difference has significant effects on the chemical and biological properties of these two amino acids, since Glu is negatively charged at physiological pH, while Gln is positively charged. Moreover, it is not apparent from the claims of the pending application or elsewhere what effect the R groups of Glu and Gln have on their nucleotide binding specificity. Without such information, it would not have been obvious to substitute Gln with Glu. Similarly, Ser, Thr, Val, Ala, Glu and Asn represent a subset of the class of all amino acids specified in the copending case. The claims of the copending case provide no reason that one would select one of the particular subset of amino acids specified in the present claims rather than other amino acids. Absent such a suggestion, the present claims are not obvious from the claims of the copending case.

Applicants also note that the amendments made to the claims in the response dated July 22, 2002 were unrelated to double patenting (either statutory or obviousness-type). Thus, neither the original nor amended claims provide a basis for statutory or obviousness-type double patenting.

4. Rejections under 35 USC 112, second paragraph

Claims 1(b) and 3(g) are said to be indefinite in not specifying the +6 nucleotide if the ++2 position is not Asp. However, these phrases are not recite in the pending claims. It appears that the Examiner may be referring to a copending case in which these phrases were at one time present.

Claim 2 is said not to have antecedent basis in claim 1 in that claim 1 does not reference base 4 to be anything other than A or C. In response, the elements from claim 2 have been copied into claim 1 and claim 2 has been amended to refer to the two nucleotides occupying base 4 previously specified in claim 1. Thus, effectively the scope of claims 1 and 2 has been reversed such that claim 2 is a further limitation of claim 1 rather than vice versa.

Claim 3(g) is said to be unclear as the meaning of a "small residue." This rejection has been rendered moot by deleting this phrase from the claim. It is noted that equivalent teaching is provided by the specification. It is further noted that the scientific and patent literature evidence frequent usage and understanding of the term "small amino acid" in the art. As evidence of this understanding, applicants attach a chart from Livingstone, Comput. Appl. Bio. Sci. 6, 745-56 (1993) defining what is meant by small amino acids. Further, a quick search of the USPTO issued patent database reveals over 350 patents using this terminology. Based on this guidance and common usage of the terminology in the scientific literature, a skilled artisan would have no difficulty designing a zinc finger protein binding to a target sequence according to the amended claims.

Claim 3(l) is said to be indefinite because of the term "asp." However, this term is not used in this claim. Rather the term "Asp" is used. This is consistent with the nomenclature for amino acids used in other claims and in the art.

Claim 3(o) is said to be a typographic error in that the Fig. 6 teaches that position +2 is Asp when base 1 is C. However, Fig. 6 does not show nucleotide quadruplets at all, much less in which position 1 is occupied by C. Thus, it is not

understood how the Examiner finds this teaching in Fig. 6. However, a typographic error in 3(o) has been corrected.

Claim 4 has been amended to refer to claim 3 rather than any preceding claim. It is noted that this amendment had been made previously in a preliminary amendment dated November 5, 2001.

Claim 14 has been amended to delete the ";".

Claim 15 has been amended as suggested.

The amendments to the above claims cure the alleged defects in claims depending therefrom.

For these reasons, withdrawal of the rejection is respectfully requested.

5. Rejections under 35 USC 102

Claims 1-7, 9-11, 13, 15-23 and 32 stand rejected as anticipated by Choo et al.. 35 USC 102(b).

As a preliminary matter, it is noted that the patentablity of a design method should be judged from the steps of the method recited in the claims rather than from the nature of the zinc finger protein resulting from the method. An improved method of design is not obvious over a prior method of design simply because the improved method of design might result in some of the same proteins as the prior method. An improved method of design that has different methods steps than a prior method may allow design of some of the same proteins as a prior method, but in addition will also allow design of some proteins that would not result from following the prior method. If the steps in the improved methods are not suggested by those of the prior method, then the improved method is patentable notwithstanding that it may design some proteins that are the same as those from the prior method.

In the remarks that follow, applicants first reiterate the position discussed in the last response that the presently claimed methods result in design of some zinc finger proteins that are not designed by following the cited reference. Although patentability is not determined by the nature of the zinc finger proteins that result from

the method, this issue is significant in showing the error in the Examiner's apparent position that any difference between the present methods and those of Choo et al. is illusory. Applicants then explain the difference in method steps between the present claims and those of the cited reference that confer patentability of the presently claimed methods.

The Examiner apparently views any difference between the present claims and Choo et al. to be an illusion due simply to different numbering schemes for bases. The Examiner presents three numbering systems in a Table on p. 5 of the office action. The first line of the table is a quadruplet numbering system labeled "Ex. Nos." which the Examiner says she is using as the basis of rejection. The second line of the table is a triplet numbering system of Choo et al. The third line of the table is a different quadruplet numbering system that is displaced from the system "Ex. Nos." by one base. In fact, the system "Ex. Nos" is the same as the numbering system of the present application, and that shown in the figures accompanying the last response. This is also true of the correspondence between "Ex. Nos" and the 5' Nos. in the second line of the Table. Thus, insofar as the Examiner bases her analysis on the Ex. Nos. and their correspondence with 5' Nos. of Choo et al., she is assuming the same correspondence as applicants.

Elsewhere in the office action, the examiner proposes different equivalences than those described between the first two lines of the table at p.7, which are the only two lines relevant to this analysis. For example, base 2 of the present claims is equated with the 3' base of Choo et al., (see e.g., office action at p. 9, line 4). Base 1 of the present claims is also equated with the 3' base of Choo et al. (see e.g., p. 9, line 6). It is clear that the 3' base of Choo et al. cannot be both base 1 and base 2 of a quadruplet. In short, the Examiner has based her comparison of rules from Choo et al. on the erroneous assumption that base 1 of the present numbering corresponds to a 3' base in Choo et al. system. Applicants note numerous other areas of disagreement with the Examiner's position that all the rules of the present claims are disclosed by Choo et al., but in view of the overriding error in assigning equivalence between base 1 of the present

claims and the 3' base of Choo et al. believe it unnecessary to address these other issues at this time.

Finally, when addressing applicants' previous remarks (office action at p. 10), the office action states that base 4 of a quadruplet (i.e., present numbering) is equivalent to the 5' base of a triplet as in Choo et al. The office action does not expressly say what alignment is proposed between the other bases in a quadruplet and those of Choo et al. In any event, if base 4 of a quadruplet is viewed as being aligned with the 5' base of a Choo et al. triplet, it follows that bases 3 and 2 of the quadruplet are aligned with the mid and 3' bases of the Choo et al. triplet, and base 1 (present numbering) is aligned with the 5' base of a different Choo et al. triplet. This alignment is shown in Fig. 3A (attached to this Response, which is the same as the upper portion of Fig. 1 previously submitted with the Response mailed July 22, 2002). As the Examiner can see, the alignment of bases in Fig. 3A is the same as that shown between Ex. Nos. and 5' Nos. in the Table at p. 7 of the office action.

Comparison of Fig. 3A (in which present base 4 is treated as being the 5' base of a Choo et al. triplet) with Fig. 3B (in which base 4 is treated as being part of a quadruplet) shows the consequential effects in design of a zinc finger protein.[1] In Fig. 3A (illustrating the Choo et al. design rules), the "G" residue is not taken into account in the design of a zinc finger. By contrast, in Fig. 3B (illustrating the presently-claimed design rules), the "G" occupies position 1 of a quadruplet, thereby specifying that the amino acid at position +2 of zinc finger F1 is a Glu. The resulting designs differ in that the +2 position of finger F1 is occupied by a Ser using the triplet design rules and a Glu using the quadruplet design rules. Therefore, besides being a different method, the use of a quadruplet code compared to a triplet code can have a material effect on the resulting design of zinc finger proteins.

The preceding analysis shows that the presently claimed methods can result in different designs of zinc finger proteins that the cited Choo et al. reference.

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[1] Figs. 3A and 3B are not part of the application but are attached to this response to illustrate differences between the claimed invention and cited reference.

Such is significant in showing that any difference between the presently claimed methods and Choo et al. is not merely an illusion resulting from a different numbering scheme.

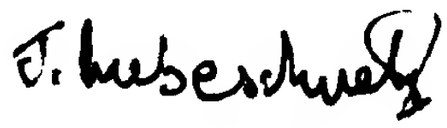
Applicants now return to the key issue in determining patentability, which is that the steps of the claimed methods are not disclosed or suggested by the cited Choo et al. reference. The presently claimed methods include steps of selecting a quadruplet of bases within a target sequence and then applying design rules to each of the four bases in the quadruplet. By contrast, the Choo et al. reference, at best, selects a triplet of bases and applies design rules to certain triplet sequences. It is not sufficient, for establishing obviousness, to say that following the Choo et al. reference may result in some of the same zinc finger proteins as following the presently claimed methods, because this position bases patentability on the zinc finger proteins that result from the methods rather than the method steps themselves. Absent evidence that Choo et al. discloses or suggests the steps recited in the present claims, the rejection should be withdrawn.

6. Rejection under 35 USC 103

Claims 1-7, 9-11, 13-23 and 32 stand rejected as obvious over Choo et al. et al in further view of Krizek. Krizek is cited as teaching the peptide sequence of SEQ ID NO:6 of the present application. Choo et al. is applied as above. In response, Krizek does nothing to remedy the lack of disclosure in Choo et al. regarding selecting quadruplets and the consequences of doing so, as discussed above. Therefore, claims 1-7, 9-11, 13-23 and 32 are not obvious for at least the reasons discussed above.

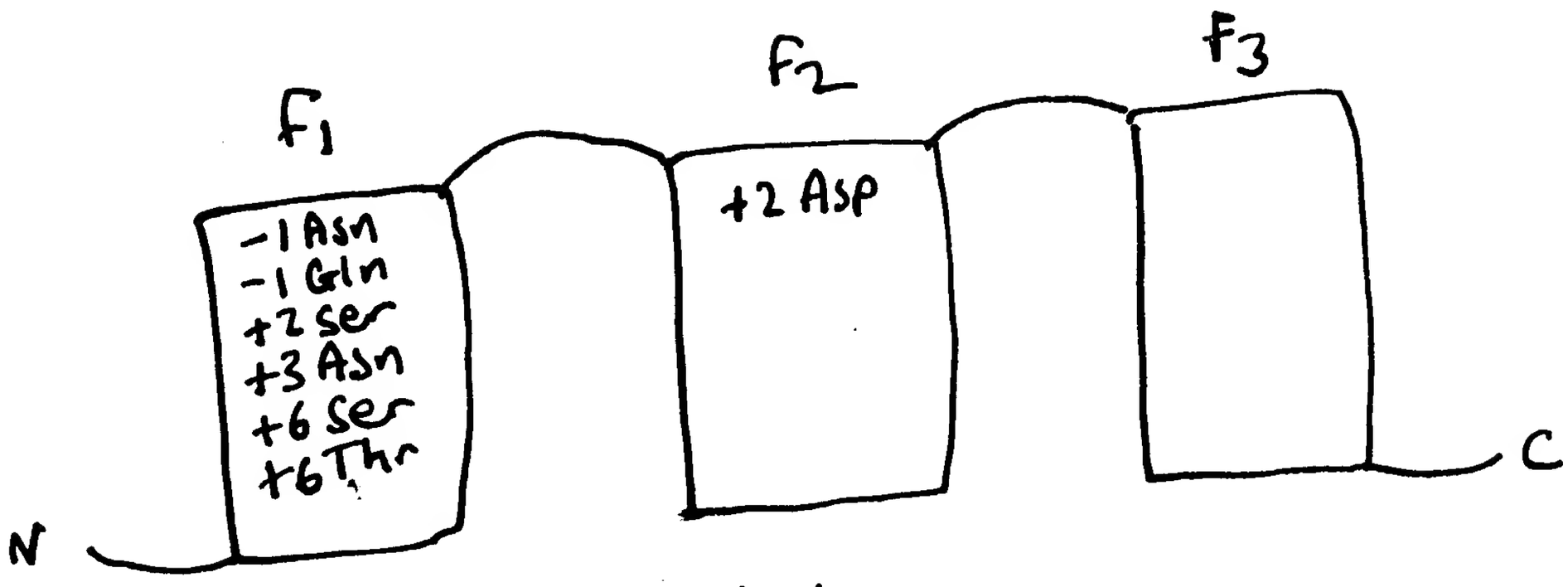
If the Examiner believes a telephone conference would aid in the prosecution of this case in any way, please call the undersigned at 650-326-2400.

Respectfully submitted,



Joe Liebeschuetz
Reg. No. 37,505

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, 8th Floor
San Francisco, California 94111-3834
Tel: 650-326-2400
Fax: 415-576-0300
JOL:jol
PA 3288823 v1

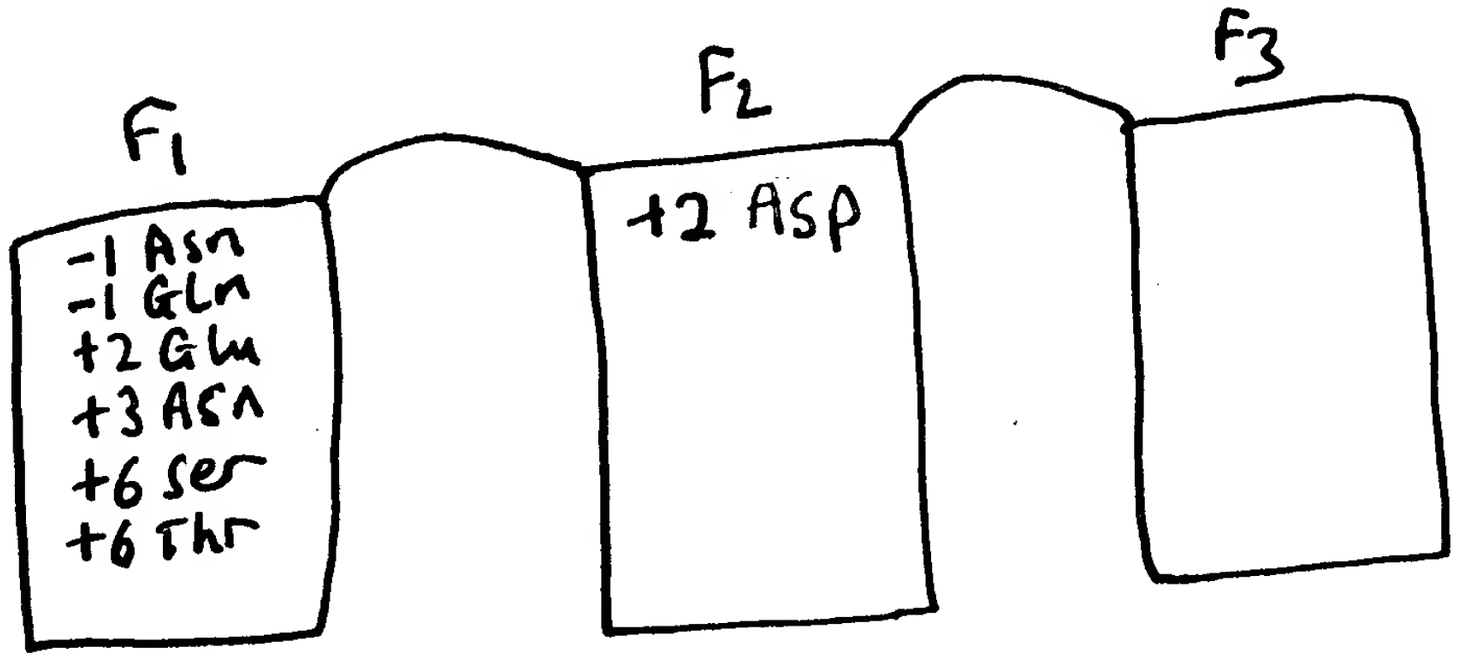


5′ 3′ mid 5′ Choo et al. numbering

G T A T

1 2 3 4 Present numbering

Fig. 3A



G T A T

1 2 3 4 Present numbering

Fig. 3B

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Protein sequence alignments: a strategy for the hierarchical analysis of residue conservation

Craig D.Livingstone and Geoffrey J.Barton[1]

Abstract

An algorithm is described for the systematic characterization of the physico-chemical properties seen at each position in a multiple protein sequence alignment. The new algorithm allows questions important in the design of mutagenesis experiments to be quickly answered since positions in the alignment that show unusual or interesting residue substitution patterns may be rapidly identified. The strategy is based on a flexible set-based description of amino acid properties, which is used to define the conservation between any group of amino acids. Sequences in the alignment are gathered into subgroups on the basis of sequence similarity, functional, evolutionary or other criteria. All pairs of subgroups are then compared to highlight positions that confer the unique features of each subgroup. The algorithm is encoded in the computer program AMAS (Analysis of Multiply Aligned Sequences) which provides a textual summary of the analysis and an annotated (boxed, shaded and/or coloured) multiple sequence alignment. The algorithm is illustrated by application to an alignment of 67 SH2 domains where patterns of conserved hydrophobic residues that constitute the protein core are highlighted. The analysis of charge conservation across annexin domains identifies the locations at which conserved charges change sign. The algorithm simplifies the analysis of multiple sequence data by condensing the mass of information present, and thus allows the rapid identification of substitutions of structural and functional importance.

Introduction

A protein that exhibits key biological functions will commonly have homologues sequenced from many different tissues and organisms. Accurate multiple sequence alignment of such a protein family can highlight the residues of common functional and structural importance. The location of identities and conservative substitutions may be used to guide the design of site-directed mutagenesis experiments whilst the identification of subtle patterns of residue conservation can yield improvements in the accuracy of secondary and tertiary structure predictions (Crawford, *et al.*, 1987; Zvelebil *et al.*, 1987; Benner and Gerloff, 1990; Barton *et al.*, 1991; Russell *et al.*, 1992). Such analyses of multiple sequence alignments have traditionally been performed by eye. However, for large alignments, only the most obvious patterns of residue conservation can be easily identified by this method. When many long sequences are to be scrutinized, the task becomes unmanageable, and the risk of missing interesting residue substitutions is great.

A number of computer programs have been developed to aid the interpretation of multiple sequence alignments. The programs PRETTY and PRETTYPLOT from the GCG package (Devereux *et al.*, 1984) derive consensus amino acid sequences and box the largest group of similar residues at each position of an alignment. ALSCRIPT (Barton, 1993) allows shading, boxing and colouring to be applied to an alignment. Colour is also exploited by the SOMAP program (Parry-Smith and Attwood, 1991), which colours residues according to which user-defined set they belong (e.g. hydrophobic, charged). The amino acid variation at a position in an alignment is reduced to a single figure of 'variability' by Kabat (1976), 'entropy' or 'variation' by Sander and Schneider (1991), 'information' by Smith and Smith (1990) and 'evolutionary divergence' by Brouillet *et al.*, (1992). In contrast, the novel set-based approach described by Taylor (1986), defines the minimal set of physico-chemical properties that represent any group of amino acids. This principle has been developed by Zvelebil *et al.* (1987) so that the minimal set of amino acids could be encoded as a single 'conservation number' at each position in the alignment. Although very effective at highlighting the overall similarity at each position in an alignment, none of these methods deal with the problem of quantifying similarities between subfamilies within a larger multiple sequence alignment.

It is frequently desirable to subdivide a protein family on the basis of function, origin, sequence similarity or other criteria. Indeed, most multiple alignment methods (e.g. Barton, 1990; Barton and Sternberg, 1987; Feng and Doolittle, 1987; Higgins and Sharp, 1989) first compare all sequences pairwise, then automatically cluster the sequences into subfamilies on the basis of sequence similarity. Such cluster analysis can readily identify the gross similarities between sequences but does not pinpoint the residue positions that are responsible for the clustering pattern. It may also be difficult to rationalize the clusters identified by overall sequence similarity with those implied by functional similarity since functional differences may reside in a few key residues. Although all previous methods for characterizing residue conservation (e.g. Kabat, 1976; Devereux

Laboratory of Molecular Biophysics, University of Oxford, Rex Richards Building, South Parks Road, Oxford OX1 3QU, UK

[1]To whom correspondence should be addressed

et al., 1984; Taylor, 1986; Smith and Smith, 1990; Parry-Smith and Attwood, 1991; Sander and Schneider, 1991; Brouillet *et al.*, 1992) provide a clear overview of conservation across an alignment, they do not allow the automatic identification of residue positions specific to subgroups of sequences within the alignment.

In this paper we describe an algorithm for the systematic identification of residue conservation within aligned protein sequences. The algorithm operates in a hierarchical manner, by first characterizing conservation on a residue-by-residue basis within predefined subfamilies, then between all pairs of subfamilies. This hierarchical approach highlights positions that may be responsible for conferring the specific structural and functional properties of the subfamilies.

Systems and methods

The hierarchical conservation analysis algorithm is implemented in the computer program AMAS (Analysis of Multiply Aligned Sequences) written in ANSI-C. AMAS can generate commands for the ALSCRIPT program (Barton, 1993), which will automatically shade, box and colour a multiple alignment according to the identified conservation patterns. AMAS and ALSCRIPT have been used successfully on a number of Unix platforms. If the graphical display options are required, then a Postscript printer or interpreter is required.

Algorithm

Quantification of amino acid residue conservation

We have extended the work of Zvelebil *et al.* (1987) to give a general method for quantifying residue conservation. Our approach differs in detail to that described by Zvelebil *et al.*, so for the sake of completeness and to avoid possible confusion we here describe the protocol used to quantify and compare residue conservation.

Figure 1(a) illustrates a Venn diagram (for details see Taylor, 1986) which is contained within a boundary that symbolizes the universal set of 20 common amino acids (ϵ). The amino acids that possess the dominant properties—hydrophobic, polar and small (<60 Å^3)—are defined by their set boundaries. Subsets contain amino acids with the properties aliphatic (branched sidechain non-polar), aromatic, charged, positive, negative and tiny (<35 Å^3). Shaded areas define sets of properties possessed by none of the common amino acids. The Venn diagram may be simply encoded as the property table or index shown in Figure 1(b), where the rows define properties and the columns refer to each amino acid.

Cysteine occurs at two different positions in the Venn diagram. When participating in a disulphide bridge (C_{S-S}), cysteine exhibits the properties 'hydrophobic' and 'small'. In addition to these properties, the reduced form (C_{S-H}) shows polar character and fits the criteria for membership of the 'tiny' set.

When analysing proteins that do not have disulphides, an index which represents the properties of reduced cysteine is used (see SH2 domain analysis). In proteins where disulphide bonding is known to occur, or where the oxidation state of the cysteines is uncertain, an index representing cysteine in the oxidized form is generally more useful (as in Figure 1b).

The illustrated Venn diagram (Figure 1a) assigns multiple properties to each amino acid; thus lysine has the property hydrophobic by virtue of its long sidechain as well as the properties polar, positive and charged. Alternative property tables may also be defined. For example, the amino acids might simply be grouped into non-intersecting sets labelled, hydrophobic, charged and neutral.

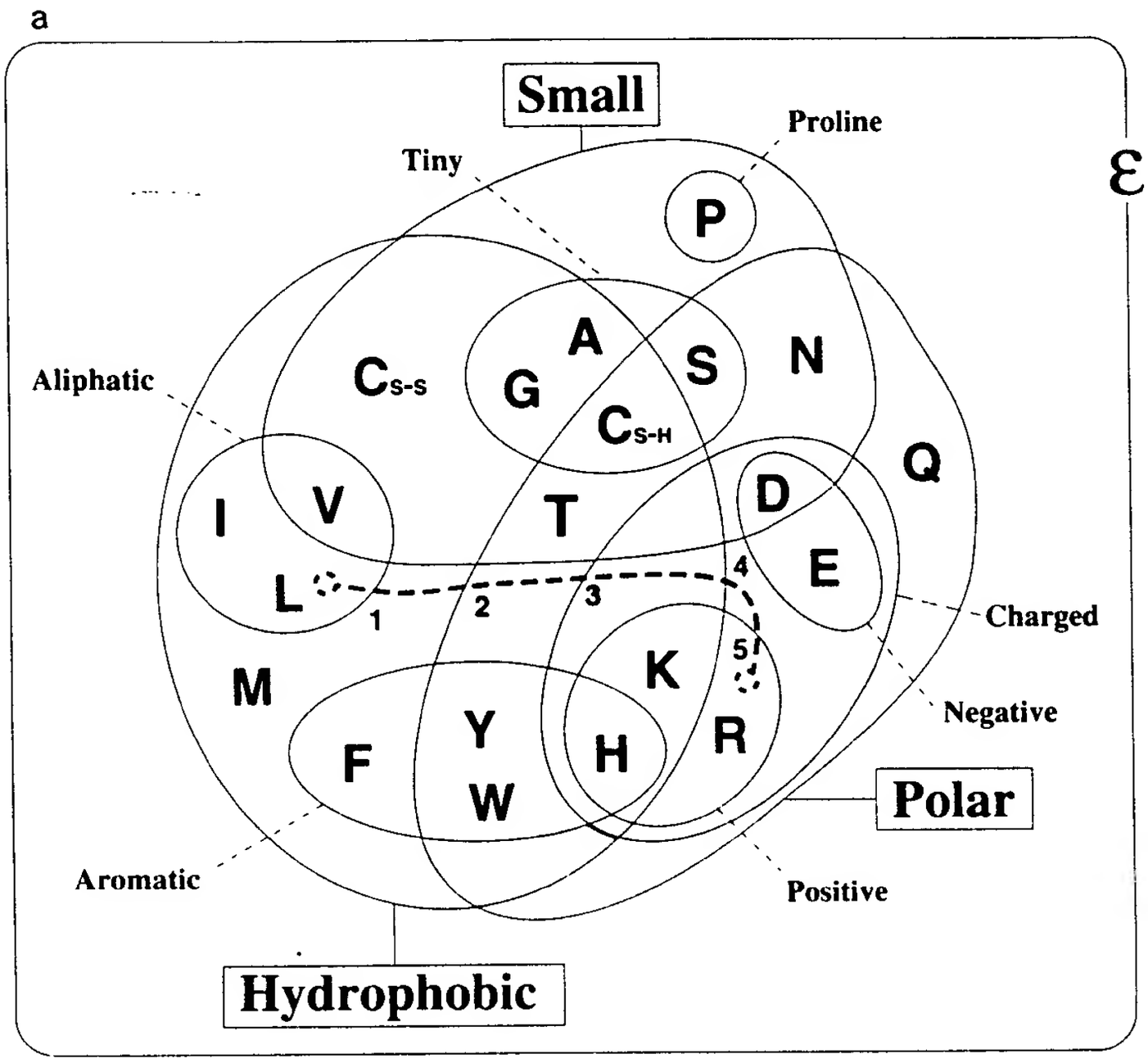
Figure 2 illustrates the stages involved in the calculation of conservation numbers for a simplified property index (Figure 2a and b). All of the amino acids are assigned to the universal set (ϵ), which in this simple example contains only the charged subset, which in turn is broken down into subsets containing positively and negatively charged amino acids. This property index allows the positions of conserved charges to be identified, together with positions where a conserved charge changes polarity between different groups of sequences within an alignment.

The amino acids occurring at each position in the multiple alignment are recorded (Figure 2d), then tested for the presence of each of the three properties (Figure 2b). This is represented by the columns of entries for each amino acid (Figure 2e). For example, at aligned position 11, the first column in Figure 2(e) represents the properties of arginine, the second column the properties of tryptophan and so on. Filled circles show the amino acid is a member of a property set, empty circles indicate non-membership.

Each property is considered in turn by examining the rows of entries in Figure 2(e). If all of the amino acids at a position possess the property, then the position shows positive conservation; all entries on that property's row in Figure 2(e) will be filled circles and a filled circle appears in Figure 2(f). If all amino acids at a position lack the property, then the position shows negative conservation; all entries on the row in Figure 2(e) will be empty circles and an empty circle is seen in Figure 2(f). If the possession of a property varies in the set of amino acids being considered, filled and empty circles appear in the equivalent row in Figure 2(e), the property is labelled as unconserved and a shaded circle is shown in Figure 2(f).

Two methods are used to quantify conservation at an alignment position using the information stored in Figure 2(f). Method 1 is similar to that of Zvelebil *et al.* (1987) and regards as conserved any property that is either positively or negatively conserved. The number properties obeying this rule (number of filled or empty circles for a position in Figure 2f) is summed

a



b

ILVCAGMFYWHKREQDNSTPBZXΔ

1	●●●●●●●●●●○○○○○○●○○○●●	Hydrophobic
2	○○○○○○○○●●●●●●●●●●○●●●●	Polar
3	○○●●●●○○○○○○○○○●●●●●○○●●	Small
4	○○○○○○○○○○○○○○○○○○●○○●●	Proline
5	○○○○●●○○○○○○○○○○○●○○○●●	Tiny
6	●●●○○○○○○○○○○○○○○○○○●●	Aliphatic
7	○○○○○○○●●●●○○○○○○○○○○●●	Aromatic
8	○○○○○○○○○○●●●○○○○○○○○●●	Positive
9	○○○○○○○○○○○○○●○●○○○○○●●	Negative
10	○○○○○○○○○○●●●●○●○○○○○●●	Charged

Fig. 1. Physico-chemical properties of the amino acids. (**a**) The 20 common amino acids are shown in terms of 10 physico-chemical properties (Taylor, 1986; Zvelebil *et al.*, 1987). Grey-filled areas define sets of properties possessed by none of the common amino acids. The hydrophobic, polar and small sets dominate the figure. The remaining sets define subsidiary groups. The dotted line joining L to R shows the minimum number of five set boundaries which must be crossed in order to change an L to an R in this 10 property diagram (see text). (**b**) An amino acid property index derived from the Venn diagram in (a) (after Zvelebil *et al.* (1987), treating Cys as C_{S-S}). The columns represent the amino acids while rows represent properties. Filled circles show when an amino acid possesses a property. Δ represents gap which, in this index, is regarded as having all properties.

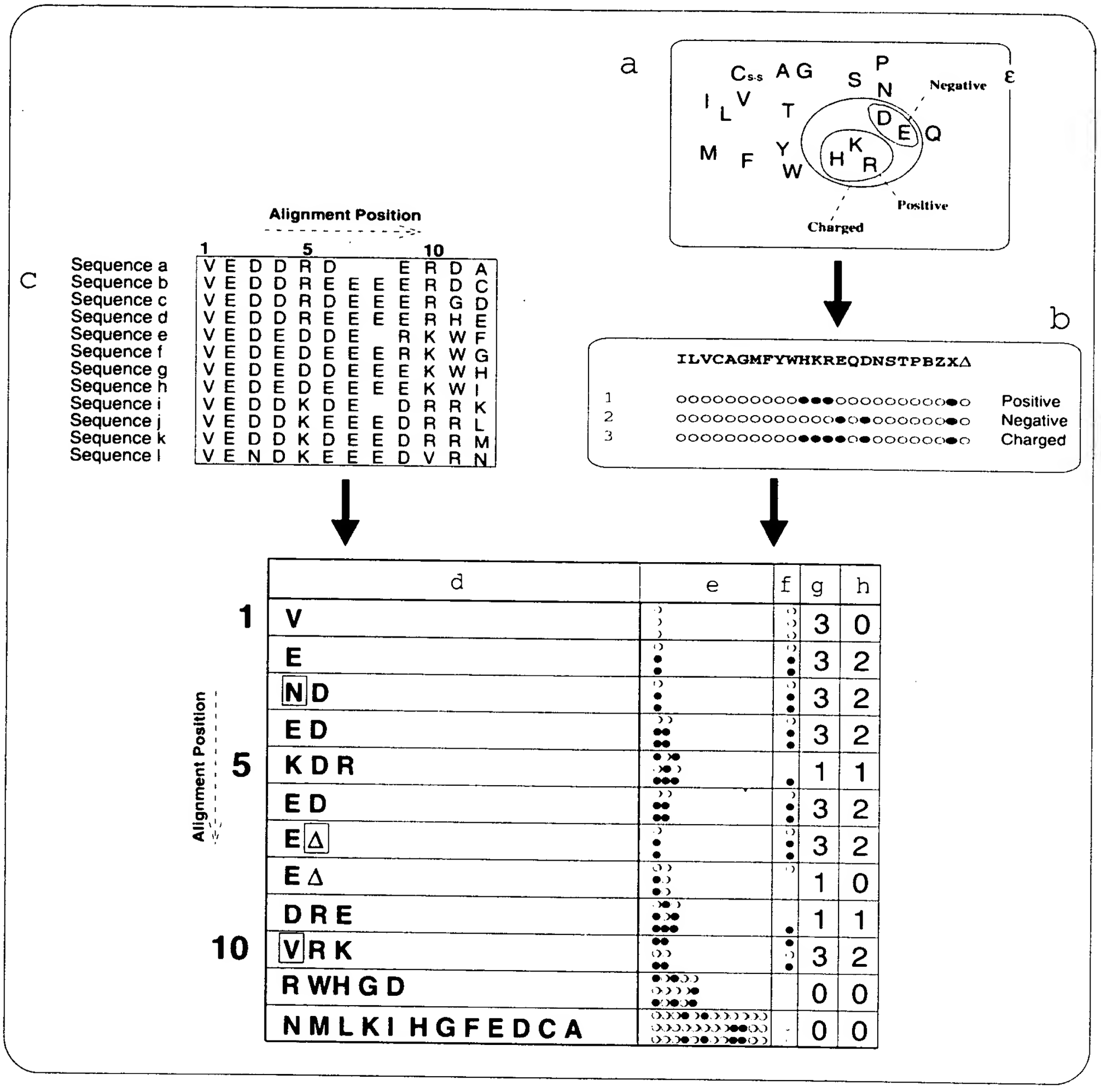


Fig. 2. Calculation of conservation numbers. The Venn diagram showing the relationship between the amino acids on the basis of charge (**a**) is converted to a property index (**b**), which is used to analyse the conservation of charged residues in the sequence alignment (**c**). The amino acids present at each sequence position are recorded (**d**) and tested for each of the properties in the index (**e**). Columns of filled (presence of a property) and empty (lack of a property) circles record the properties of each amino acid in the same vertical order as in the property index. The presence of properties is summed (**e**), filled circles show positive conservation of a property in the group of amino acids, shaded circles show where properties are present in some but not all of the amino acids, and empty circles show negatively conserved properties. A conservation score is arrived at by summing either the number of positively and negatively conserved properties (**g**—method 1) or the number of positively conserved properties alone (**h**—method 2) (see text).

to give the conservation number (Figure 2g). In contrast, method 2 only counts properties that are positively conserved (filled circles in Figure 2f) and gives the conservation numbers shown in Figure 2(h).

The method 1 conservation value is a function of the number of set boundaries P that must be crossed to visit all the amino acids at a position. If a property index contains N properties then the conservation number (C_n) is $N - P$. For example, the dotted line in Figure 1(a) joins Leu and Arg and crosses five set boundaries, thus for this property matrix, $C_n(L,R) = 10 - 5 = 5$. The maximum possible value for the conservation number calculated by method 1 is given by the number

of properties in the property index (3 for Figure 2b; 10 for Figure 1b).

Conservation by method 2 is calculated by counting the number of sets common to all amino acids at a position. Leu and Arg in Figure 1(a) share no properties; by method 2, their conservation number is 0. Asp and Glu in Figure 2(a) are both members of the sets charged and positive; their conservation number by method 2 is 2. The maximum value for the conservation value calculated by method 2 is the maximum number of properties possessed by a single amino acid in the property index.

Treatment of gaps and unusual residues

Insertions and deletions (gaps—Δ) are usually tolerated only in surface loop regions. Accordingly, gaps are normally given all properties in the property matrix so that aligned positions that contain a gap are assigned a low conservation value.

The set-based conservation analysis described here is independent of the number of sequences analysed. For example, a position in an alignment of 100 sequences that contains 99 alanines and one lysine will give the same conservation value as a position in an alignment of two sequences that has one alanine and one lysine. The advantage of this approach is that the tolerance of particular physico-chemical properties at a position indicates the likely environment of the amino acids in the common fold of the protein family. This reasoning suggests that a position that conserves valine in 99 sequences, but also shows aspartate is unlikely to be performing a common structural or functional role. However, it may sometimes be suspected that one or more of the sequences contain errors, or that there are errors in the alignment. It is then desirable to relax the strict conservation rules. Accordingly, a predetermined number of gaps or residues that represent $<N\%$ of the total at a position may be ignored when calculating conservation values. For example, alignment position 3 in Figure 2 is predominantly Asp. This position would not be recorded as conserved using the charge index due to the presence of a single Asn (1/12 or 8.3% of the sequences in the alignment). If a 10% threshold for unusual residues is set, then this Asn would be ignored when calculating the conservation value (similarly, Val at position 10). Positions where unusual residues have been ignored are reported only as conserved, never as identical even if the other residues present are identical (Figure 2, position 3). It is the ability to quantify the conservation of amino acids that gives the set-based approach its major advantage over averaging a single property scale, caution must therefore be exercised when deciding to ignore gaps and unusual residues.

Hierarchical conservation analysis

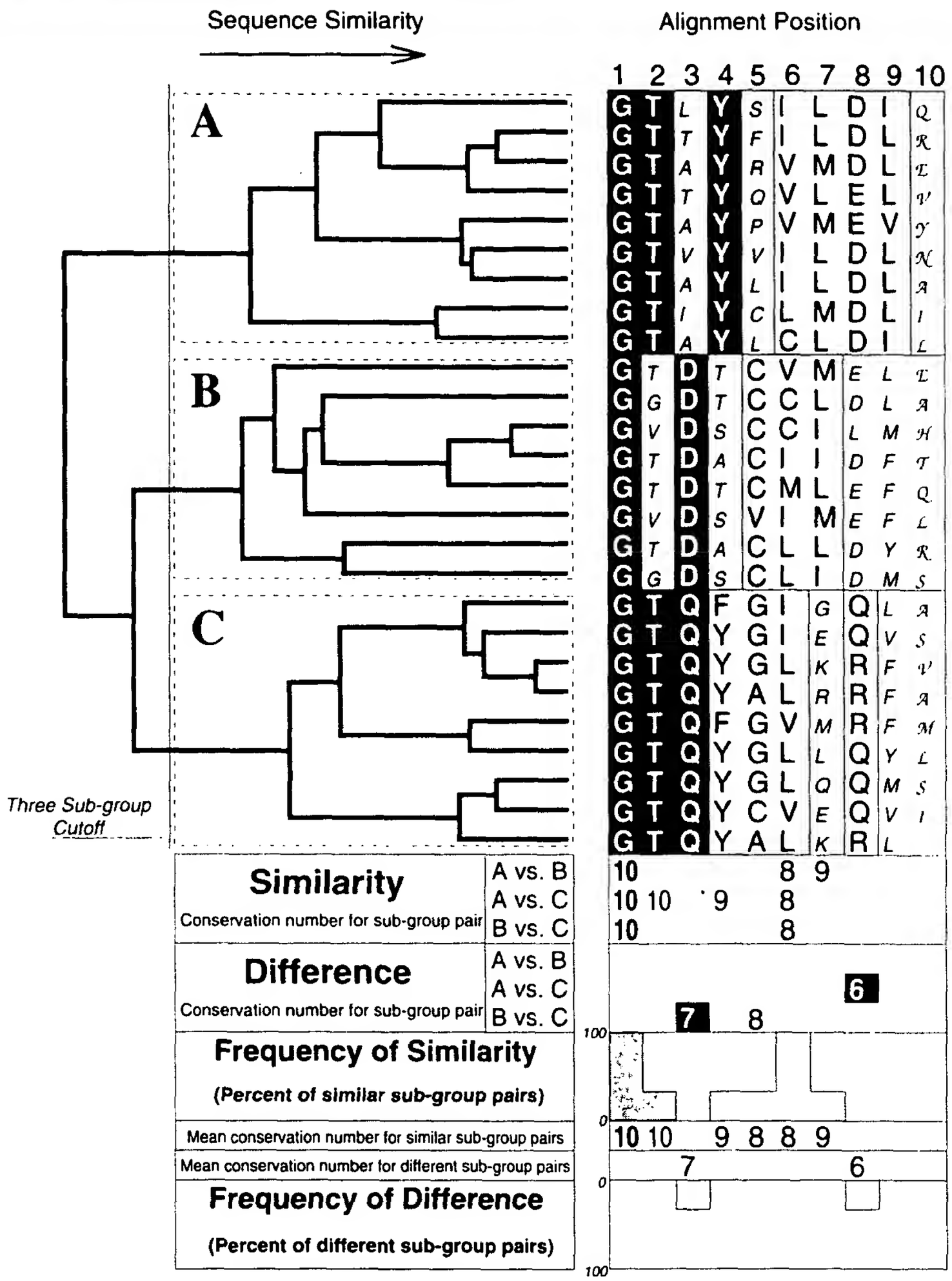
The procedures described in the previous section are a straightforward extension of the principles described by Zvelebil *et al.* (1987) and Taylor (1986). Here we extend the set-based method to identify conserved features of sequence subgroups within larger protein sequence alignments.

The starting point for hierarchical conservation analysis is the identification of two or more subsets of sequences within a multiple sequence alignment. The subsets may be defined by grouping on the basis of overall sequence similarity, by functional similarity, origin or other criteria. Given such groupings, the aim is to highlight which residue positions define the unique properties of each group.

Figures 3 and 4 illustrate the result of applying hierarchical conservation analysis to a nine residue fragment of a 26 sequence multiple alignment using the 10 property index shown in Figure 1. The dendrogram shown at the left of Figure 3 shows the overall similarity between the sequences (i.e. not just the nine residues) and clearly splits the sequences into three subgroups labelled A, B and C.

Conservation numbers are calculated for each alignment position in each subgroup and a conservation threshold is set. This reference point is used to put each position within a sub-group into one of three classes: (i) identical positions; (ii) conserved positions, where the conservation number is greater than or equal to the threshold; and (iii) unconserved, where the conservation number is less than the threshold. The choice of threshold depends upon the particular conservation index being used. For the index shown in Figure 1, a threshold of between 6 and 8 normally gives the most informative results. In Figure 3, the different classifications using a threshold of 8 are illustrated by shading and font changes. For example, in subgroup A, identities are shown in white on dark grey at positions 2 and 4, conserved positions are in black on light grey (positions 6–9), and unconserved positions are illustrated in italics on a white background (positions 3 and 5). At position 1, the identity in all sequences is marked by white on black lettering, whilst at position 10 chancery script lettering is used to highlight the lack of conservation within all sub-groups.

Having classified the conservation within each subgroup, all pairs of subfamilies are compared and conservation numbers calculated for each position in the pairs. In the calculation of conservation for a pair of subfamilies, the residues from the pair are considered as members of a single group. C_n is then calculated, as described above, for the composite group according to which method was chosen. The change in conservation value that occurs when each pair of subfamilies is brought together reflects the similarities or differences in physico-chemical properties seen in each subgroup at that position. For example, at position 7 of subfamilies A and B the conservation values in A, B and A + B are 9, showing that the properties are conserved within each family, and across both families at this position. This is, therefore, a location that exhibits common physico-chemical properties between A and B, yet these properties are not conserved within group C. Accordingly, this may indicate a tertiary structural feature shared between A and B, but not C.



KEY

D	Identical across all sequences
Y	Identical within one sub-group
Q	Conserved within one sub-group
E	Unconserved within one sub-group
S	Unconserved across all sequences

Fig. 3. Hierarchical conservation analysis. A 10 residue fragment of a multiple sequence alignment of 26 sequences is shown to the right of the figure. The relationship between the sequences in the whole alignment is represented by the dendrogram to the left, which shows three sub-groups: A, B and C. Each position of the groups in the multiple sequence alignment has been analysed for residue conservation using the property index in Figure 1(b). The conservation threshold was set to 8. Information about the conservation pattern is given at the foot of the alignment in numerical and graphical form. The representation of the alignment and the conservation patterns to the right of the figure were imported directly from the graphical output of the programm AMAS.

Conservation Threshold: 8 (Maximum: 10)
Number of gaps ignored: 0
Percentage of unusual residues ignored: 0%

Sub-group A, sequences: 1 to 9
Sub-group B, sequences: 10 to 17
Sub-group C, sequences: 18 to 26

❶

Identity between all sub-groups

(1) ALL [G]

Identity between sub-group pairs

(2) A,C [T]

Identity within one sub-group

(3) B [D] C [Q]
(4) A [Y]

❷

Conservation between all sub-groups

								%	%	%
(6) ALL	(8, 8, 8)	A-[ILVC]	B-[ILVCM]	C-[ILV]	Cons: Hydrophobic	Diff: Small	A-[44]	B-[37]	C-[22]
						Aliphatic	A-[88]	B-[62]	C-[100]

Conservation between sub-group pairs

							%	%	
(4)	A,C	(9)	A-[Y]	C-[FY]	Cons: Hydrophobic Aromatic	Diff: Polar	A-[100]	C-[77]	
(7)	A,B	(9)	A-[LM]	B-[ILM]	Cons: Hydrophobic	Diff: Aliphatic	A-[66]	B-[75]	

Difference between sub-group pairs

							%	%	
(3)	B,C	(7)	B-[D]	C-[Q]	Cons: Polar	Diff: Small	B-[100]	C-[0]	
						Negative	B-[100]	C-[0]	
						Charged	B-[100]	C-[0]	
(5)	B,C	(8)	B-[VC]	C-[CAG]	Cons: Hydrophobic Small	Diff: Tiny	B-[0]	C-[88]	
						Aliphatic	B-[12]	C-[0]	
(8)	A,C	(6)	A-[ED]	C-[RQ]	Cons: Polar	Diff: Small	A-[77]	C-[0]	
						Positive	A-[0]	C-[44]	
						Negative	A-[100]	C-[0]	
						Charged	A-[100]	C-[44]	

Conserved within one sub-group

(9) A (9) A-[ILV] Cons: Hydrophobic Aliphatic

❸

Unconserved within one sub-group

(2) B (7) B-[TGV]
(3) A (6) A-[LTAVI]
(4) B (7) B-[TSA]
(5) A (1) A-[SFRQPVL]
(7) C (2) C-[GEKRMLQ]
(8) B (4) B-[EDL]
(9) B (7) B-[LMFY] C (6) C-[LVFYM]

Unconserved across all sub-groups

(10) A (1) A-[QREVYNAIL] B (1) B-[EAHTQLRS] C (0) C-[ASVMLIΔ]

Fig. 4. Text representation of sequence conservation. With reference to Figure 3. The text representation of the analysis gives a more detailed description of the conservation of physico-chemical properties at each alignment position. Each record identifies the sequence position to which it refers (rounded brackets), the sub group(s) involved in the pattern being reported, the pair conservation number(s) of those groups where non-identities are reported (rounded brackets), the residues present in each group (square brackets) and the properties which are conserved by them and which differ between them. Differences in properties between subgroups are reported; the percentage of residues in each subgroup that have a property is shown in square brackets.

In contrast, at position 8 of subgroups A and C, in order to 'visit' all members of the combined set of amino acids from A + C (DEQR) a minimum of four set borders must be crossed, giving a value of C_n as 10 — 4 =6. The conservation values for A, C and A + C are, therefore 9, 8 and 6 respectively. Thus, although properties are conserved within each subgroup

at this position, the properties that are conserved differ between the subgroups. This type of conservation pattern might highlight a position in the protein structure that defines the specificity for a substrate. For example, the switch from a predominantly negative to positive charge between groups A and C may signal increased binding for a negatively charged moiety for the group C sequences when compared to group A.

General rules for linking such substitution patterns to changes in three-dimensional structure or function are as yet unknown. However, changes in conservation of charge, hydrophobicity or amino acid size are likely to be of importance in all protein families.

The result of the pairwise comparison of subfamilies is summarized below the alignment in Figure 3. The conservation values for the pairs of subgroups are either displayed as similarities of differences according to the rules shown in Table I. The similarity and difference sections are also summarized as histograms.

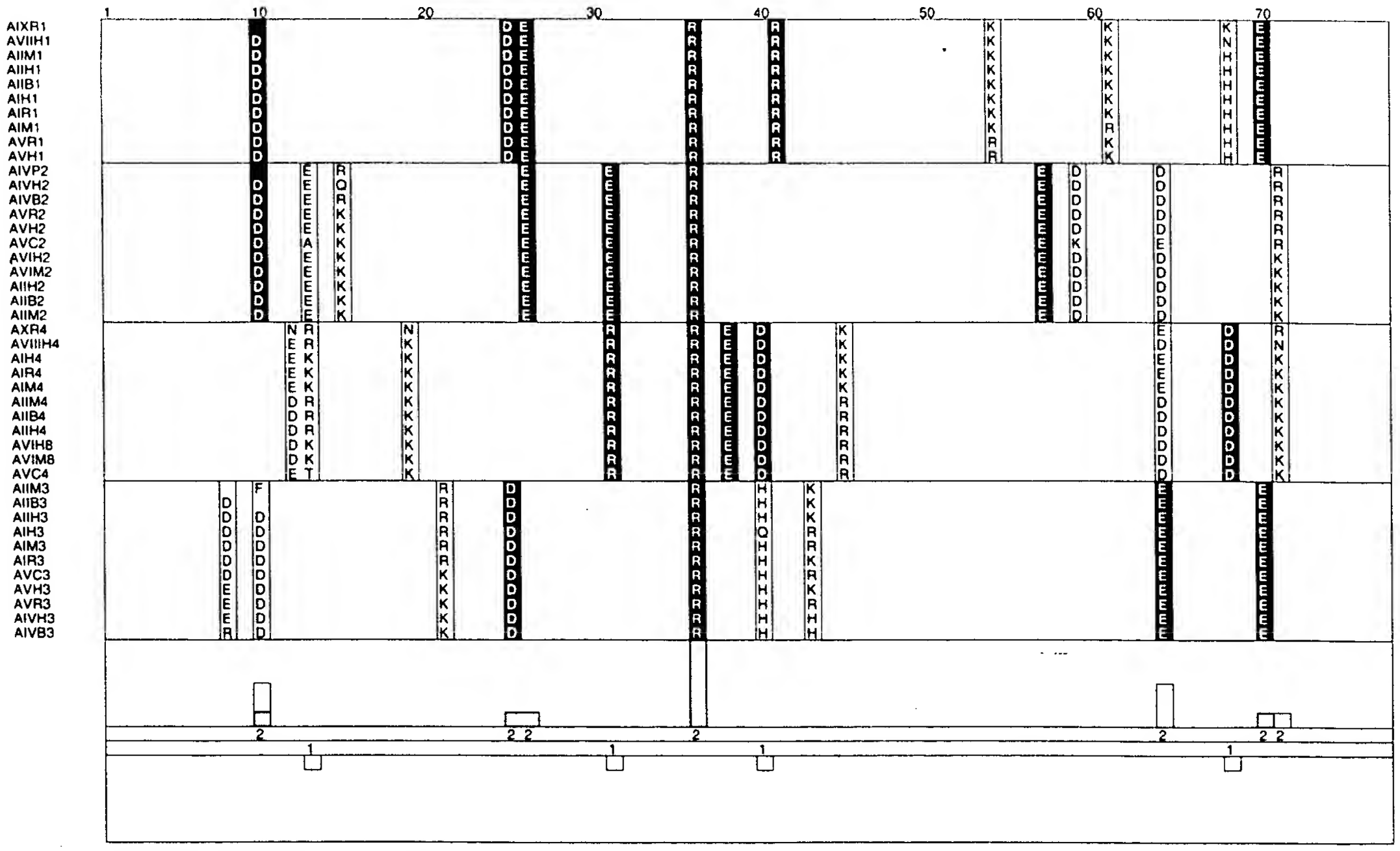
The hierarchical clustering approach addresses the problem of how to weight the information content of each sequence in

Table I . Pair comparison of conserved sequence subgroups

Sub-groups compared			Display C_{A+B} as
A	B	A + B	similarity/difference
$C_A \geq T$	$C_B \geq T$	$C_{A+B} \geq \min C_A, C_B$	similarity
$C_A \geq T$	$C_B \geq T$	$C_{A+B} < \min C_A, C_B$	difference but conserved
$C_A \geq T$	$C_B \geq T$	$C_{A+B} < T$	difference and unconserved
$C_A < T$	$C_B \geq T$	–	–
$C_A \geq T$	$C_B < T$	–	–
$C_A < T$	$C_B < T$	–	–

Conservation values are calculated for the sub-groups A and B, and for the sub-groups combined A + B. A conservation threshold T is set, similarities or differences are reported according to the logical operations shown.

a



AMAS conservation plot of the sequence alignment newest

(Stringency is set to 85% with 3 property matrix /home/orac/cdl/birch.pf)

b

Conservation Threshold: 2 (Maximum: 3)
Number of gaps ignored: 2
Percentage of unusual residues ignored: 12%

Sub-group A, sequences: 1 to 10
Sub-group B, sequences: 23 to 33
Sub-group C, sequences: 45 to 55
Sub-group D, sequences: 67 to 77

❶

Identity between all sub-groups

(36) ALL [R]

Identity between sub-group pairs

(10) A,B [D]
(25) A,D [D]
(26) A,B [E]
(70) A,D [E]

Identity within one sub-group

(31) B [E] C [R]
(38) C [E]
(40) C [D]
(41) A [R]
(57) B [E]
(64) D [E]
(68) C [D]

❷

Conservation between all sub-groups

NONE

Conservation between sub-group pairs

(10)	A,D (2) A-[D] D-[D]	Cons: Negative Charged	Diff:	
	B,D (2) B-[D] D-[D]	Cons: Negative Charged	Diff:	
(64)	B,C (2) B-[D] C-[ED]	Cons: Negative Charged	Diff:	
	B,D (2) B-[D] D-[E]	Cons: Negative Charged	Diff:	
	C,D (2) C-[ED] D-[E]	Cons: Negative Charged	Diff:	
(71)	B,C (2) B-[KR] C-[K]	Cons: Positive Charged	Diff:	

Difference between sub-group pairs

					%	%
(13)	B,C (1) B-[E] C-[KR]	Cons: Charged	Diff: Positive	B-[0]	C-[90]
			Negative	B-[90]	C-[0]
(31)	B,C (1) B-[E] C-[R]	Cons: Charged	Diff: Positive	B-[0]	C-[100]
			Negative	B-[100]	C-[0]
(40)	C,D (1) C-[D] D-[H]	Cons: Charged	Diff: Positive	C-[0]	D-[90]
			Negative	C-[100]	D-[0]
(68)	A,C (1) A-[H] C-[D]	Cons: Charged	Diff: Positive	A-[90]	C-[0]
			Negative	A-[0]	C-[100]

Conserved within one sub-group

(8)	D (2) D-[ED]	Cons: Negative Charged	
(12)	C (2) C-[ED]	Cons: Negative Charged	
(15)	B (2) B-[KR]	Cons: Positive Charged	
(19)	C (2) C-[K]	Cons: Positive Charged	
(21)	D (2) D-[KR]	Cons: Positive Charged	
(43)	D (2) D-[HKR]	Cons: Positive Charged	
(45)	C (2) C-[KR]	Cons: Positive Charged	
(54)	A (2) A-[KR]	Cons: Positive Charged	
(59)	B (2) B-[D]	Cons: Negative Charged	
(61)	A (2) A-[K]	Cons: Positive Charged	

Fig. 5. Charge conservation in 40 annexin repeats. (**a**) The pattern of conserved charge in 40 annexin repeats determined using the charge property index described in Figure 2. Only positive property conservation is considered at a conservation threshold of 2, this means that a subgroup position must conserve both charge and polarity to be reported. Conserved positions alone are reported in order to highlight the pattern of charged residues; the residues at unconserved positions have been masked out. Two gaps, and residues constituting $< 10\%$ of a subgroup position have been screened from the conservation calculation. Identities and conserved positions are identified according to the shading protocol given in Figure 3. A charge difference is clearly seen in the histogram at position 31, reflecting the switch between a conserved E (negative) in repeat 2 and a conserved R (positive) in repeat 4. (**b**) Text output accompanying the analysis in Figure 6(a). The record format used is identical to that used in Figure 4.

an alignment. At the simplest level, each sequence would be treated equally but this relies on the sequences being equally diverse throughout the alignment. The use of clustering to derive conservation patterns ensures equal weight is given to different groups of proteins irrespective of the number of examples of each type. Inevitably, this process involves the loss of informa-

tion about the minor sequence variation which is responsible for subtle differences in character similar proteins in a subgroup. This loss is balanced by the ability to detect the more substantial changes in conservation which determine the differences in properties between the separate subgroups.

Implementation

Text representation

AMAS accepts command line arguments and provides a detailed textual breakdown of the conservation within a multiple alignment. Figure 4 illustrates the AMAS textual analysis that corresponds to the alignment shown in Figure 3. Only those positions that display conservation of the properties in the chosen property index are described. The presentation of the text results is hierarchical. Identities are described first (1), followed by positions showing conservation of physico-chemical properties (2), and unconserved positions listed last (3). Each entry contains a record of the alignment position (rounded brackets to the left), of the subgroup(s) to which it refers and a list of the residues in each subgroup cited (square brackets). In addition for positions that do not show identities, the properties conserved at the position, and those that differ are reported. With reference to Figure 4:

- *Identities.* Section 1 lists those sequence positions that are identical across the whole alignment, between pairs of subgroups and within one subgroup. Information is not repeated lower down the hierarchy if it has already been presented, e.g. the Gly at position 1 in the alignment is not also reported as two pairs of identical subgroups or as three identical individual subgroups.
- *Conservation of properties.* Conservation of physico-chemical properties between subgroups (following the same redundancy rules as for identities) is reported in section 2. The four categories of conserved positions are: (1) all subgroups conserve similar properties; (2) pairs of conserved subgroups share properties; (3) pairs of conserved subgroups have dissimilar properties; and (4) individual subgroups are conserved. The properties that are positively conserved between pairs of subgroups are listed, as are those properties that cause differences between subgroups. For each of a pair of different subgroups, the percentage of residues that display the differing properties is shown in square brackets.
- *Unconserved.* There are two divisions, the first for single unconserved subgroups and the second for entirely unconserved alignment positions.

Graphical display

The optional graphical representation of results mimics a hand analysis of the alignment using coloured marker pens. In Figure 3 the alignment is shown divided into three subfamilies. Within the subfamilies, at each alignment position, the amino acids are appropriately highlighted. Conserved subgroups, subgroups showing identity and positions that show identity across the whole alignment are labelled. Figures 5 and 6 illustrate the graphical representation applied to the annexin and SH2 domains.

Three highlighting methods have been explored. Monochrome methods allow grey shading (Figure 5 and 6) or the use of different fonts (not shown) to highlight the differences in conservation. Grey shading is preferable for publication, whilst unshaded alignments are useful as working copies for hand annotation. Colour may be specified as an alternative to shading to provide additional visual impact.

Discussion

The strategy described in this paper is extremely flexible: it allows different physico-chemical properties to be examined independently, or in concert. In addition, an alignment may be dissected into any combination of subgroups and their relative conservation analysed. As with any analytical procedure, the strategy is most effective when one has a clear idea of what one is looking for. For example: 'What makes subgroup A different from B and C?', or 'Which residues in subgroup D should I change to make D more like A?' If no clear questions have been defined, then the general property index (Figure 1b) is a useful starting point to highlight patterns of residue conservation. This is illustrated in Figure 6 for an alignment of 67 SH2 domains (Russell *et al.*, 1992). Since SH2 domains are cytoplasmic, Cys was assigned the properties of the free amino acid (C_{S-H}) in this analysis (Figure 1b). The alignment is divided into eight subgroups on the basis of overall sequence similarity. Subgroups 1−7 (numbering from the top) share $>20\%$ sequence identity, whilst sequences not fitting into one of these subgroups are collected in subgroup 8. The overall conservation of physico-chemical properties is highlighted by the histogram at the base of the alignment. The upper histogram indicates the normalized frequency of similarities between pairs of subgroups, whilst the lower plot shows the frequency of pair differences. Dark shading of the histogram indicates the frequency of pairs of subgroups that show sequence identity. A hand analysis of an alignment similar to that shown in Figure 6 correctly identified the location of the core secondary structures, and phosphotyrosine-binding residues (Russell *et al.*, 1992; Barton and Russell, 1993). Since completion of that study, the three-dimensional structures of three SH2 domains have been determined by the techniques of X-ray crystallography and NMR. The secondary structures of these are illustrated at the base of Figure 6 (Booker *et al.*, 1992; Overduin *et al.*, 1992; Waksman *et al.*, 1992). The conservation histograms clearly correspond to the regions of secondary structure, and are helpful in identifying patterns characteristic of α-helix and β-strand. For example, at positions 15 and 97, CXXCCXXC patterns

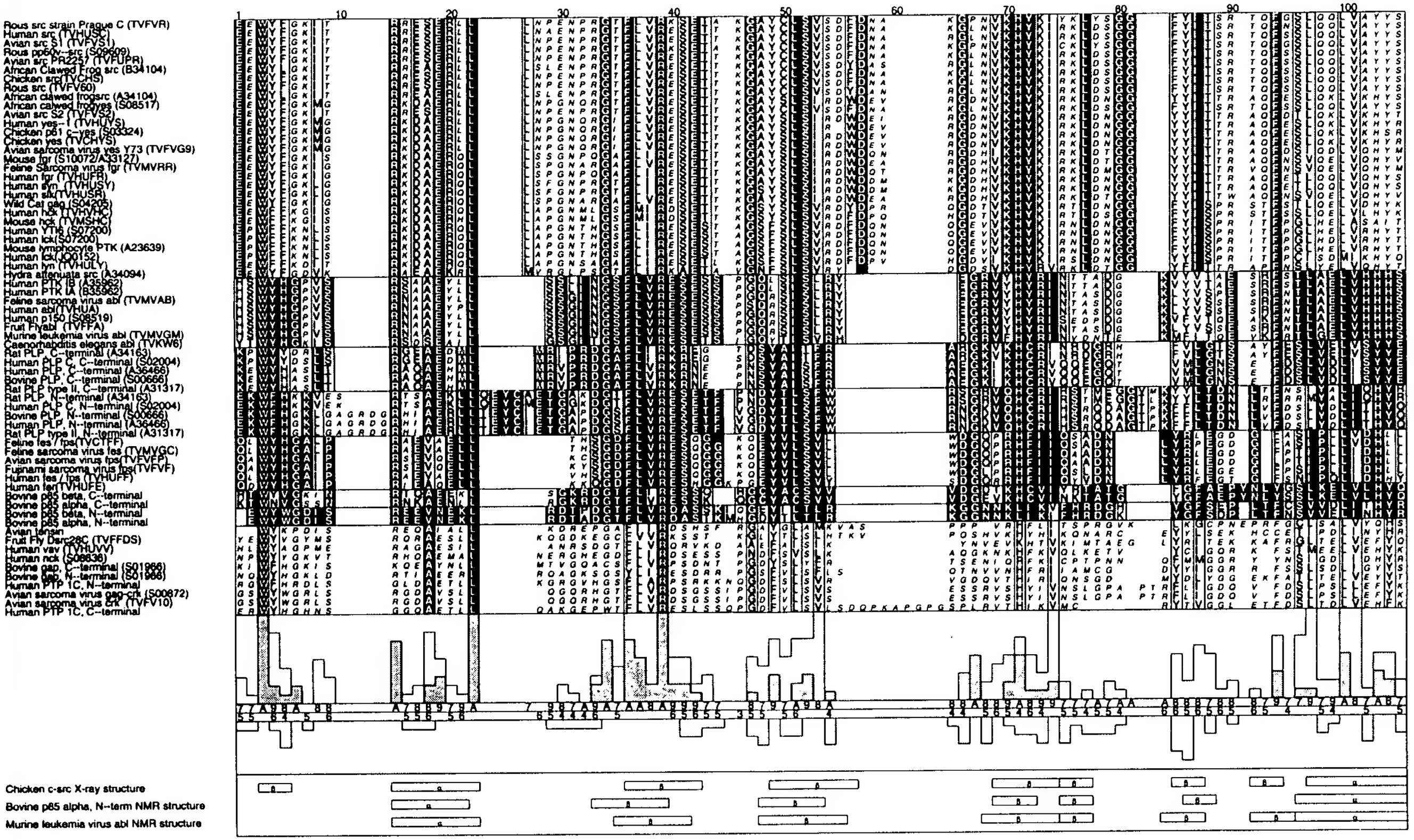


Fig. 6. Conservation analysis of 67 SH2 domains. An alignment of 67 SH2 domains analysed using the general property index (Figure 1b). A key to the shading strategy is given in Figure 3 (see text). The mean pair conservation number for conserved subgroup pairs at each position is reported below the histogram if it is equal to or exceeds the threshold of 7 for the plot. One gap per subgroup was ignored.

(where C = conserved) characteristic of α-helix are clearly visible.

The annexins are a family of proteins that bind phospholipid in a calcium-dependent manner. Annexins consist of a variable N-terminal sequence followed by four or eight repeats, each of ~80 amino acids. Inspection of a multiple sequence alignment of 40 repeats identified the unique features of each repeat family, and located patterns of residue substitution characteristic of the secondary structures (Barton *et al.*, 1991). Figure 5 illustrates the application of hierarchical conservation analysis to a subset of these annexin repeats. Only conserved charges are shown (Figure 5a), and the differences summary clearly locates the position of a change in charge sign (position 31). This charge swap corresponds to the site of an inter-repeat salt bridge (Barton *et al.*, 1991). Additional charge changes are also seen at positions 13, 31, 40 and 68 as listed in the textual summary shown in Figure 5(b). While all these features can be identified by hand inspection of the alignment, the process is laborious and error-prone. The strategy described in this paper reduces the scope for error, allows alternative subgroupings to be investigated rapidly, and provides shading and boxing that is structurally relevant.

AMAS and Alscript are available from the authors.

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